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PRINCIPAL INVESTIGATOR: Yosef Yarden, Ph.D.

CONTRACTING ORGANIZATION: The Weizmann Institute of Science
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Yosef Yarden, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

The Weizmann Institute of Science
Rehovot, 76100, Israel

e-mail:
liyarden@weizmann.ac.il

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The HER2/ErbB-2 oncogenic protein contributes to virulence of human breast cancer and serves as a useful target for cancer immunotherapy. However, the exact mechanism underlying ErbB-2 function is still unknown. Our studies of the last year have concentrated on the widely accepted possibility that ErbB-2 acts as a direct receptor for a still unknown growth factor. By screening several potential candidates we found no known growth factor that fulfills the expected attributes of an ErbB-2 specific ligand. However, this search resulted in the identification of a novel growth factor, which we named Neuregulin-4, whose direct receptor is ErbB-4 (see publication 1). Systematic comparison of all of the known ligands of ErbB proteins, including three viral molecules, led us to conclude that ErbB-2 may not function as a direct receptor for a specific ligand. Instead, our studies imply that it acts as a shared co-receptor for many, if not all, ErbB ligands (See publication 2). This conclusion explains the tumorigenic action of ErbB-2 in terms of the cross-talk between the stroma and the epithelial tumor cells.

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Introduction

The ErbB/HER family consists of four distinct members, including the epidermal growth factor receptor (EGFR or ErbB-1), ErbB-2, ErbB-3 and ErbB-4. These receptors are embedded in the plasma membrane and include four domains: an extracellular ligand binding domain, a transmembrane domain and an intracellular region, which includes a tyrosine kinase fused to a carboxy-terminal tail. Within the ErbB family the highest homology spans the tyrosine kinase and the extracellular domains, while the carboxy-terminal tail diverges. However, ErbB-3 harbors several amino acid differences in the tyrosine kinase domain, which renders it an inactive enzyme. Ligand binding to the extracellular domain of many receptor tyrosine kinases, including ErbBs, results in receptor dimerization and activation of the tyrosine kinase. Autophosphorylation by the activated kinase on tyrosine residues located at the carboxy-terminal tail is the last step in receptor activation. Different effector proteins are then recruited to the tyrosine-phosphorylated residues, thus activating downstream signaling pathways leading to growth, differentiation, or other cellular responses.

Multiple ligands exist to the ErbB receptors: ErbB-1-specific ligands include the epidermal growth factor (EGF), transforming growth factor alpha, amphiregulin and the heparin-binding EGF-like protein. Neuregulins (NRGs), a family of four different genes with multiple splice variants, encode ligands for ErbB-3 and/or ErbB-4. Betacellulin and epiregulin, which were isolated as ErbB-1-specific ligands, are bound also by ErbB-4. A ligand specific to ErbB-2 has not been isolated to date and thus, under physiological conditions ErbB-2 homodimers probably do not form. However, ErbB-2 is the preferred heterodimeric partner of the other members of the family, ErbB-1, -3 and –4, and its overexpression leads to oncogenic transformation. In addition, co-expression of ErbB-2 together with ErbB-1, or one of the NRG receptors, exerts a synergistic effect on cell transformation. Accordingly, heterodimers with ErbB-2 generate a stronger proliferative signal compared with their respective homodimeric forms. The formation of a specific heterodimer is probably governed by ligand identity and the relative expression levels of individual receptors. Remarkably, heterodimerization that recruits ErbB-3 into active dimers can establish signaling complexes whose mitogenic and morphogenic signals are relatively high due to the tail of ErbB-3, which is endowed with a large variety of docking sites.

Although ErbB-2 shares extensive structural homology with other ErbBs along both the catalytic intracellular domain and in the extracellular putative ligand binding region, many attempts

to identify stimulatory ligands specific to ErbB-2 have so far failed. Thus, several candidate ligands isolated from T cells, human mammary carcinoma cells and a rat adenocarcinoma have not been completely characterized. In addition, detection of an activity that enhances ErbB-2 phosphorylation led to molecular cloning of the Neu differentiation factor (NDF) and heregulin, two of a dozen isoforms of neuregulin 1, all of which bind to ErbB-3 and ErbB-4. Nevertheless, several observations imply that ErbB-2 homodimers, the plausible outcome of a direct ligand, may be functional in vivo. For example, an oncogenic mutation that activates ErbB-2 phosphorylation apparently stabilizes such homodimers, and bivalent monoclonal anti-ErbB-2 antibodies are mitogenic because they, like a direct ligand, dimerize ErbB-2 on the cell surface.

In parallel with attempts to isolate a direct ligand, several approaches culminated at the possibility that ErbB-2 may function, at least in part, as a co-receptor. Thus, co-expression of ErbB-2 together with ErbB-1 enhanced EGF-induced mitogenesis and its presence reconstituted an extremely potent proliferative activity of ErbB-3, which is totally inactive when singly expressed. Consistent with its transactivating capability, ErbB-2 was found to act as the preferred partner of ligand-driven ErbB heterodimers. The use of intracellular antibodies to ErbB-2 has led to the conclusion that it can enhance signaling by two growth factors, EGF and NDF, through an ability to decelerate their release from the direct receptors, namely ErbB-1 and either ErbB-3 or ErbB-4, respectively. By using mutant ErbB molecules and a chimeric EGF/NDF ligand we reached the conclusion that ErbB-2 acts as a low affinity receptor of NDF, once this ligand is immobilized at the cell surface by one of its high affinity receptors (see our previous annual report). According to this model, which was supported by in vitro studies performed with soluble ErbB-1 and ErbB-2 molecules, ErbB ligands are bivalent molecules that recruit ErbB-2 through their low-affinity and broad-specificity binding site.

Does ErbB-2 function as a high affinity receptor for a still unknown ligand of the EGF/neuregulin families, or could it act solely as a shared receptor subunit that amplifies signaling by prolonging the action of heterologous ligands? Our studies of the last year have focused on this question by using two approaches. First, we screened all available genomic databases for EGF and NRG-like ligands in the hope of identifying a direct ErbB-2 ligand. This work resulted in the identification of a novel ligand of ErbB-4, but no ErbB-2-specific ligand has been found. Second, we examined the generality of the transactivation ability of ErbB-2 by combining most existing

ErbB ligands with monoclonal antibodies (mAbs) that block the putative ligand binding site of ErbB-2. Our results strongly support the possibility that ErbB-2 evolved as a pan-EGF/neuregulin receptor rather than a high affinity receptor for a novel ligand.

Body

1. Identification of Neuregulin-4: a novel ligand of ErbB-4 (see Publication 1 for details)

The ErbB/HER family of receptor tyrosine kinases consists of four receptors that bind a large number of growth factor ligands sharing an epidermal growth factor- (EGF-) like motif. Whereas ErbB-1 binds seven different ligands whose prototype is EGF, the three families of neuregulins (NRGs) activate ErbB-3 and/or ErbB-4. Here we characterize a fourth neuregulin, NRG-4, that acts through ErbB-4. The predicted pro-NRG-4 is a transmembrane protein carrying a unique EGF-like motif and a short cytoplasmic domain. A synthetic peptide encompassing the full-length EGF-like domain can induce growth of interleukin-dependent cells ectopically expressing ErbB-4, but not cells expressing the other three ErbB proteins or their combinations. Consistent with specificity to ErbB-4, NRG-4 can displace an ErbB-4-bound NRG-1 and can activate signaling downstream of this receptor. Expression of NRG-4 mRNA was detected in the adult pancreas and weakly in muscle; other tissues displayed no detectable NRG-4 mRNA. The primary structure and the pattern of expression of NRG-4, together with the strict specificity of this growth factor to ErbB-4, suggest a physiological role distinct from that of the known ErbB ligands.

2. Does ErbB-2 acts in tumor cells solely as a co-receptor for stromal ligands? (see Publication 2 for details)

The *erbB-2/HER2* oncogene is overexpressed in a significant fraction of human carcinomas of the breast, ovary and lung in a manner that correlates with poor prognosis. Although the encoded protein resembles several receptors for growth factors, no high affinity ligand of ErbB-2 has so far been fully characterized. However, several lines of evidence have raised the possibility that ErbB-2 can augment signal transduction initiated by binding of certain growth factors to their direct receptors. Here we contrasted these two models of ErbB-2 function: First, examination of a large series of epidermal growth factor- (EGF-) like ligands and neuregulins, including virus-encoded ligands as well as related motifs derived from the pro-EGF molecule, failed to detect interactions with ErbB-2 when this protein was singly expressed. On the other hand, by using antibodies that

block inter-ErbB interactions and cells devoid of surface ErbB-2, we learnt that signaling by all ligands, but those derived from pro-EGF, were enhanced by the oncoprotein. These results imply that ErbB-2 evolved as a shared receptor subunit of all ErbB-specific growth factors. Thus, oncogenicity of ErbB-2 in human epithelia may not be due to the existence of a specific ligand but to the ability to act as a co-receptor for multiple stroma-derived growth factors.

Conclusions

Our studies of the last year provide explanations to several important issues related to the function of ErbB-2/HER2 in breast cancer. Instead of considering this oncoprotein as a specific receptor for a single growth factor, we suggest that ErbB-2 has lost its high affinity ligand and acquired broad specificity to multiple stromal ligands affecting tumor growth. According to this explanation, overexpression of ErbB-2 in a large fraction of breast tumors augments their virulence because it can bias recruitment of ErbB-2 into heterodimers with the respective specific receptors. In addition, our studies provide an explanation to the existence of a catalytically inactive receptor, namely ErbB-3. This receptor must interact with the co-receptor (ErbB-2) in order to transmit signals. Beyond the implications to cancer biology, our results imply that ErbB-2 is a non-autonomous oncoprotein. In other words, its harmful effect in tumors critically depends on stromal ligands and at least one other ErbB protein. This realization may open the way for new therapeutic strategies directed to ErbB ligands, as well as to the other receptors of the ErbB family, including ErbB-3.

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Neuregulin-4: a novel growth factor that acts through the ErbB-4 receptor tyrosine kinase

D Harari[1], E Tzahar[1], J Romano[1], M Shelly[1], JH Pierce[2], GC Andrews[3] and Y Yarden*,[1]

[1]*Department of Biological Regulation, The Weizmann Institute of Science, Rehovot 76100, Israel;* [2]*The National Cancer Institute, Bethesda, Maryland 20892, USA; and* [3]*Pfizer Central Research, Groton, Connecticut 06340, USA*

The ErbB/HER family of receptor tyrosine kinases consists of four receptors that bind a large number of growth factor ligands sharing an epidermal growth factor- (EGF)-like motif. Whereas ErbB-1 binds seven different ligands whose prototype is EGF, the three families of neuregulins (NRGs) activate ErbB-3 and/or ErbB-4. Here we characterize a fourth neuregulin, NRG-4, that acts through ErbB-4. The predicted pro-NRG-4 is a transmembrane protein carrying a unique EGF-like motif and a short cytoplasmic domain. A synthetic peptide encompassing the full-length EGF-like domain can induce growth of interleukin-dependent cells ectopically expressing ErbB-4, but not cells expressing the other three ErbB proteins or their combinations. Consistent with specificity to ErbB-4, NRG-4 can displace an ErbB-4-bound NRG-1 and can activate signaling downstream of this receptor. Expression of NRG-4 mRNA was detected in the adult pancreas and weakly in muscle; other tissues displayed no detectable NRG-4 mRNA. The primary structure and the pattern of expression of NRG-4, together with the strict specificity of this growth factor to ErbB-4, suggest a physiological role distinct from that of the known ErbB ligands.

Keywords: growth factor; oncogene; pancreas; signal transduction; tyrosine kinase

Introduction

Cell-to-cell signaling is an essential feature of multicellular organisms, playing important roles in both the unfolding of developmental diversification as well as mediating the homeostasis of vastly different cell types. A large number of tyrosine kinase growth factor receptors play key roles in this process. Type-1 tyrosine kinase receptors, also known as ErbB/HER proteins, comprise one of the better-characterized families of growth factor receptors, of which the epidermal growth factor receptor (ErbB-1) is the prototype (reviewed in Burden and Yarden, 1997). The ErbB family constitutes four known receptors which dimerize upon ligand stimulation, transducing their signals by subsequent autophosphorylation catalyzed by an intrinsic cytoplasmic tyrosine kinase, and recruiting downstream signaling cascades.

The ErbBs are activated by a large number of ligands. Depending upon the activating ligand, most homodimeric and heterodimeric ErbB combinations can be stabilized upon ligand binding (Tzahar *et al.*, 1996), thus allowing a complex, diverse downstream signaling network to arise from these four receptors. The choice of dimerization partners for the different ErbBs, however, is not arbitrary. Spatial and temporal expression of the different ErbBs do not always overlap *in vivo*, thus narrowing the spectrum of possible receptor combinations that an expressed ligand can activate for a given cell type (Erickson *et al.*, 1997; Gassmann *et al.*, 1995; Lee *et al.*, 1995; Pinkas-Kramarski *et al.*, 1997; Riethmacher *et al.*, 1997). Furthermore, a hierarchical preference for signaling through different ErbB receptor complexes takes place in a ligand-dependant manner. Of these, ErbB-2-containing combinations are often the most potent, exerting prolonged signaling through a number of ligands, likely due to an ErbB-2-mediated deceleration of ligand dissociation (Karunagaran *et al.*, 1996; Tzahar *et al.*, 1996; Wang *et al.*, 1998). In contrast to possible homodimer formation of ErbB-1 and ErbB-4, for ErbB-2, which has no known direct ligand, and for ErbB-3, which lacks an intrinsic tyrosine kinase activity (Guy *et al.*, 1994), homodimers either do not form or are inactive. Heterodimeric ErbB complexes are arguably of importance *in vivo*. For example, mice defective in genes encoding either NRG-1, or the receptors ErbB-2 or ErbB-4, all result in identical failure of trabeculae formation in the embryonic heart, consistent with the notion that trabeculation requires activation of ErbB-2/ErbB-4 heterodimers by NRG-1 (Gassmann *et al.*, 1995; Lee *et al.*, 1995; Meyer and Birchmeier, 1995).

At the biochemical level, the known ErbB ligands fall into several categories (Riese *et al.*, 1996b). One category, the ErbB-1 ligands, includes EGF, transforming growth factor α (TGFα), epiregulin, amphiregulin, betacellulin and the heparin-binding EGF (HB-EGF) (Higashiyama *et al.*, 1991; Marquardt *et al.*, 1984; Shing *et al.*, 1993; Shoyab *et al.*, 1989; Toyoda *et al.*, 1995). To different extents, these ErbB-1 binding ligands can also activate other receptors of the ErbB family, and hence may mediate distinct signaling outputs for a given cell type (reviewed in Tzahar and Yarden, 1998). Another category of ErbB ligands consists of the Neuregulin (NRG) family. NRG-1 (also named Neu differentiation factor (NDF), heregulin, glial growth factor, and acetylcholine receptor inducing activity) was first identified by its ability to indirectly phosphorylate ErbB-2 (Holmes *et al.*, 1992; Peles *et al.*, 1992; Wen *et al.*, 1992). Subsequently, NRG-1 was found to directly bind ErbB-3 and ErbB-4 and to sequester ErbB-2 by receptor dimerization (Peles *et al.*, 1993; Plowman *et al.*, 1993; Sliwkowski

*Correspondence: Y Yarden
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et al., 1994; Tzahar *et al.*, 1994). Multiple isoforms of NRG-1 exist which amongst other roles, permit heterogeneous binding affinities to different ErbB complexes (Tzahar *et al.*, 1994). The NRG family now includes also two isoforms of NRG-2 (Busfield *et al.*, 1997; Carraway *et al.*, 1997; Chang *et al.*, 1997; Higashiyama *et al.*, 1997), of which the alpha isoform is a pan-ErbB ligand (Pinkas-Kramarski *et al.*, 1998), and NRG-3, a ligand of ErbB-4 (Zhang *et al.*, 1997). The multiplicity of genes encoding ErbB-1 ligands, contrasting with the small number of known genes encoding ligands for ErbB-3 or ErbB-4 (namely: NRGs), led us to believe in the existence of additional NRG genes in the genome of mammals. Here we report on a fourth neuregulin, denoted NRG-4, which acts through the ErbB-4 receptor tyrosine kinase. In addition to its novel structure, this growth factor displays a pattern of expression different from other EGF-like molecules.

Results

Identification of a candidate novel ErbB ligand

With the assumption that there may still exist novel ErbB-specific ligands we decided to search for new family members by homology. The recent explosion of DNA sequencing data added to DNA databases, largely resultant from the Human Genome Project initiative, offers scanning of these data for novel transcripts coding ligands with homology to the ErbB-3- and ErbB-4-specific ligand, NRG-1 (NDF). The motif $CX_7CXNGGXCX_{13}CXCX_3YXGXRC$, conserved in most isoforms of NRG-1, was used to scan available new DNA sequences. An expressed sequence tag (EST) clone originating from a mouse liver cDNA library (accession number AA238077) was identified, its sequence encoding an EGF-like domain sharing 32% identity with the NRG-1β isoform (Wen *et al.*, 1992). This clone was obtained and fully sequenced, its presumed translation product encoding a protein of 115 amino acids (Figure 1a). Hydropathy analysis using the Kyte-Doolittle algorithm (Kyte and Doolittle, 1982) supports the existence of a transmembrane domain (Figure 1b) characteristic to most NRG isoforms (Marchionni *et al.*, 1993; Wen *et al.*, 1994). Conspicuously, this protein sequence lacks a hydrophobic amino-terminal stretch, commonly found in signal peptide motifs, important in sequestering proteins to traverse the plasma membrane. Most isoforms of NRG-1 also lack consensus signal peptide sequences, but they carry an apolar N-terminal sequence thought to allow transmembrane orientation of the precursor molecule. The predicted extracellular domain of the precursor protein includes the EGF-like domain, whose primary structure displays the entire structural motifs characteristic to the EGF/NRG family (Figure 1c). The putative cytoplasmic domain of the precursor protein is relatively short and contains one potential site for N-glycosylation. Two additional sites are located at the probable ectodomain.

Alignment of the EGF-like domains of all known ErbB-specific ligands of mammalian origin indicated that the novel transcript encodes a new member of this family (Figure 1c). Its characteristic six extracellular cysteine residues and their conserved spacing predict the existence of the three disulfide bridges, denoted as A, B and C, that are the landmark of all EGF-like peptides. Besides the six conserved cysteine residues, the new EGF-like domain shares very high homology with other members of the NRG family, including a glycine at position 21 (Gly-21), Gly-42 and Arg-44, along with many semi-conserved residues. Of note, the expected B loop of the new protein, like the loops of EGF and NRG-2, is shorter by three residues. Except for the EGF-like domain and the transmembrane topology of the novel predicted protein, it shares no

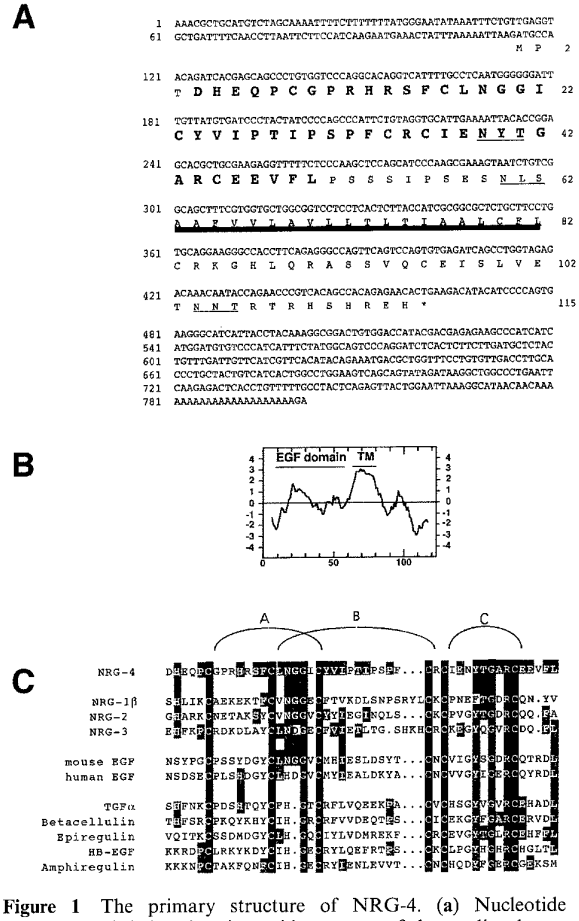

Figure 1 The primary structure of NRG-4. (**a**) Nucleotide sequence and deduced amino acid sequence of the predicted pro-NRG-4 transcript. Nucleotides are numbered at the left-hand column and amino acids at the right-hand column. The EGF-like domain with its six cysteine residues is shown in bold type, and potential N-glycosylation sites are underlined. The filled box underlines the predicted transmembrane amino acid sequence. (**b**) Hydropathy profile of pro-NRG-4. The method of Kyte and Doolittle (1982) was used with a window of 11 residues. Positive values indicate increasing hydrophobicity. Amino acid numbers are indicated below the profile. The putative transmembrane stretch of the pro-NRG-4 is marked. Note the absence of a recognizable signal peptide at the N-terminus. (**c**) Alignment of the amino acid sequence of the EGF-like domain of NRG-4 with the EGF-like motifs of other growth factors. Canonical residues are boxed in black. Other identities with NRG-4 are shaded in gray. The predicted three disulfide bonds of the motifs (Cys 1–3, Cys 2–4 and Cys 5–6) are shown above the alignment and labeled as loops A, B and C. The abbreviations used are as follows: NRG, neuregulin; TGFα, transforming growth factor α; HB-EGF, heparin-binding EGF-like growth factor. If not specified, the species of origin of all ligands is murine, except NRG-1 (rat)

significant sequence homology or structural motifs with other ErbB ligands.

Tissue-specific expression of the novel transcript

Northern blot analysis of mRNA isolated from different human adult tissues revealed moderate expression of the novel transcript in skeletal muscle and high levels in the pancreas (Figure 2). Other tissues, including brain and placenta, two rich sources of many different growth factors, displayed very low, if any, expression. Three discernible molecular weight species (0.8, 1.8 and 3.0 kilobases) were detectable in pancreas and in muscle, indicating the existence of several mRNA isoforms, the smallest band consistent in size with the clone described in this study.

The EGF-like domain of NRG-4 stimulates proliferation of ErbB-4-expressing cells

To test the prediction that the novel transcript encodes an ErbB-specific ligand, we synthesized the corresponding full-length EGF-like domain (residues 4–50, Figure 1a), denatured and refolded the synthetic peptide to allow proper disulfide bridging. This method has been used before to synthesize functionally active derivatives of other EGF-like growth factors (Barbacci *et al*., 1995; Lin *et al*., 1988; Shelly *et al*., 1998). Previously we have established a series of derivatives of the 32D cell line engineered to express different ErbB receptors or their combinations (Pinkas-Kramarski *et al*., 1996; Shelly *et al*., 1998). The myeloid 32D parental cells require cytokine stimulation, such as interleukin 3 (IL3) for their growth, and were chosen because they lack endogenous ErbB expression. Signaling through different ErbB-receptors can replace the IL3-dependent mitogenicity and survival for these cell lines, and hence this system provides a sensitive means to detect ligand-induced growth signals, which are conveniently measured as a function of cellular metabolic activity by using the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl] tetrazolium bromide (MTT) assay (Mosman, 1983).

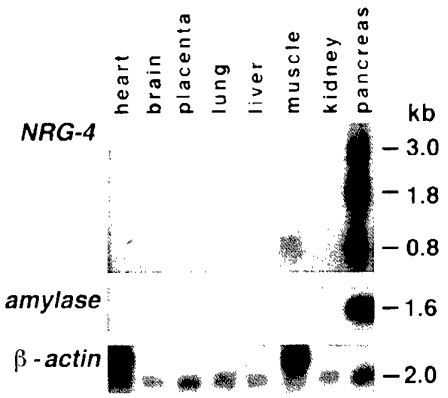

Figure 2 Northern blot analysis of NRG-4 expression in human tissues. Poly(A)-containing RNA from the indicated human tissues (2 μg per lane) was analysed using a nitrocellulose filter purchased from Clontech (San Diego, CA, USA). The blot was hybridized with a full-length NRG-4 cDNA probe radiolabeled using the Klenow fragment of DNA polymerase I and random hexamers as primers. Following autoradiography, the filter was stripped of radioactivity and re-probed sequentially with pancreas and muscle markers, *alpha-amylase-2* and *beta-actin*, respectively. Molecular weights of marker molecules are indicated in kilobases (kb). Note that *beta-actin* probe also hybridized with a larger molecular weight isoform present in heart and in skeletal muscle

Cells singly expressing ErbB-1, ErbB-2 or ErbB-3 (denoted D1, D2 or D3, respectively) did not respond to the synthetic novel peptide in a 24-h dose-response assay, although responses to EGF (D1 cells), an ErbB-2-stimulatory monoclonal antibody (D2 cells, (Klapper *et al*., 1997)), or IL-3 (D3 cells) were retained (Figure 3a, and data not shown). The latter cell line is not responsive to NRGs due to the defective kinase of ErbB-3. However, ErbB-4 expressing cells (D4), exhibited a modest dose-dependent mitogenic response in comparison to its counterpart NRG-1β control. Because different heterodimeric complexes of ErbB proteins can diversify and enhance signaling by EGF-like ligands (Cohen *et al*., 1996; Pinkas-Kramarski *et al*., 1996; Riese *et al*., 1995), cells co-expressing two ErbB proteins (for example D12 cells co-express ErbB-1 and ErbB-2) were also tested for NRG-4-induced mitogenicity. Of the tested combinations, namely: D12, D13, D23 and D24 cells, a cell line expressing a combination of ErbB-4 with ErbB-2 (D24 cells) was the only line that responded mitogenically to the novel peptide (Figure 3a). Notably, co-overexpression of ErbB-1 and ErbB-2 resulted in a relatively high basal proliferation activity, but these cells still responded to EGF (Figure 3b, and data not shown). Additionally, in cells co-overexpressing ErbB-2 and ErbB-4, NRG-1 and the novel ligand were almost equipotent (compare D4 and D24 panels in Figure 3a), indicating that ErbB-2 can enhance the mitogenic effect of the novel ligand, as it does for other ErbB ligands (Graus-Porta *et al*., 1995; Karunagaran *et al*., 1996; Wang *et al*., 1998).

A long-term cell survival assay confirmed the ability of the novel growth factor to stimulate ErbB-4. This assay examined the ability of added growth factors to sustain survival of certain 32D derivatives in the absence of IL-3. As in the dose-response experiments, the novel synthetic peptide only stimulated the survival of the two ErbB-4-expressing cell lines we examined, namely D4 and D24 cells (Figure 3b). Also similar to the short-term dose response assay, stimulation of D24 cells was more robust, and akin to the NRG-1-treated controls than was the response of D4 cells. These data indicate that the novel growth factor can exert a weak proliferative signal through ErbB-4 alone, but co-expression of ErbB-2 with ErbB-4 allows a superior mitogenic response, as it does in the case of NRG-1 (Wang *et al*., 1998). On the basis of the ability of the novel synthetic peptide to mediate a biological effect through one of the neuregulin receptors we named it neuregulin-4 (NRG-4).

NRG-4 recognizes and activates ErbB-4

To elucidate the molecular interactions pertaining to NRG-4 signaling, several different approaches were employed to test specific binding of this growth factor to the four ErbB proteins. In the first assay, binding studies in a cell-free system were performed with recombinant soluble forms of all four ErbB proteins. The soluble proteins, denoted IgB-1 through -4, consist of a dimeric fusion between the extracellular domain of the corresponding ErbB and the Fc portion of a

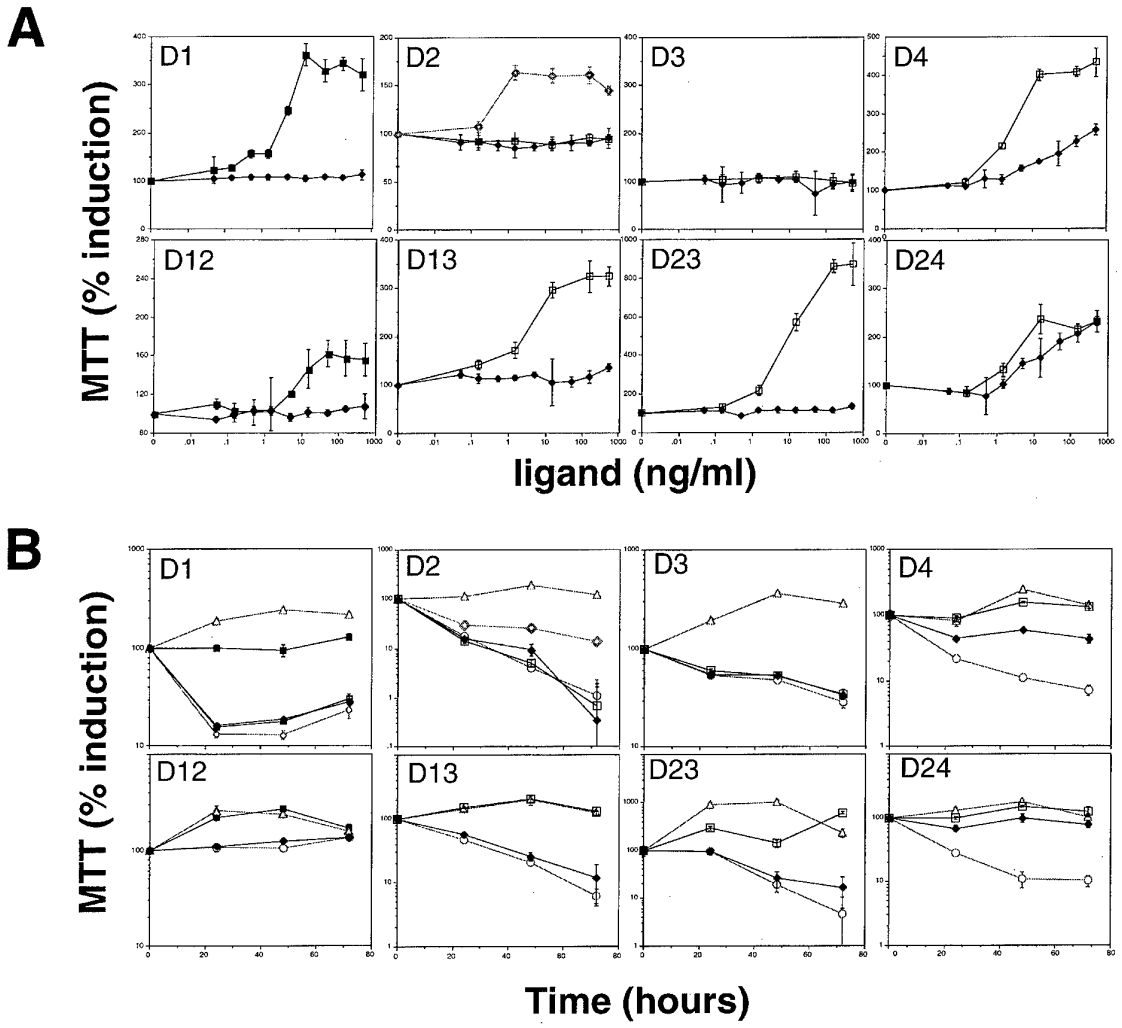

Figure 3 Proliferative and survival effects of NRG-4 on ErbB-expressing derivatives of 32D cells. (**a**) Cell proliferation assay. The indicated derivative lines of 32D cells were tested for cell proliferation using the MTT assay. Cells were deprived of IL-3 and then plated at a density of 5×10^5 cells/ml in media containing serial dilutions of NRG-4 (closed diamonds), EGF (closed squares), NRG-1β (open squares), or the L96 (maximal dose: 50 μg/ml) anti-ErbB-2 monoclonal antibody (open diamonds). The MTT assay was performed 24 h later. Results are presented as per cent induction over the control untreated cells, and are the mean$\pm$s.d. of four determinations. Each experiment was repeated at least twice with similar findings. Note that no responses to EGF-like ligands were observed with cells expressing either ErbB-2 or ErbB-3 alone, but these cell derivatives retained response to IL-3 (data not shown). (**b**) Cell survival assay. The indicated derivatives of 32D cells were incubated for various time intervals in the absence of IL-3. The following ligands, each at a concentration of 100 ng/ml, were incubated with cells: NRG-4, EGF, NRG-1β, or 50 μg/ml mAb L96 (symbols are as described in (**a**)). For control, cells were incubated with medium conditioned by IL-3-producing cells (open triangles), or with no factor (open circles). The extent of cell proliferation was determined daily by using the colorimetric MTT assay. The data presented are the mean$\pm$s.d. of four determinations. Note that co-expression of ErbB-1 and ErbB-2 (D12 cells) enabled cell survival in the absence of IL-3. The experiment was repeated twice with similar results

human immunoglobulin G (Chen *et al.*, 1996). NRG-4, EGF and NRG-1β were radiolabeled with ^{125}I, incubated with the soluble receptors, and then irreversibly bound to the IgBs using the BS^3 covalent crosslinking reagent. As expected for the controls, a strong signal was detected for EGF binding to IgB-1 in contrast to NRG-1β, which bound strongly to IgB-3 and IgB-4, but no ligand bound to IgB-2 (Figure 4a). In comparison to NRG-1, ^{125}I-NRG-4 bound to the soluble form of ErbB-4 (IgB-4) only weakly, with low or no binding to the other IgB proteins (Figure 4a). To confirm specificity of the covalent crosslinking assay we co-incubated unlabeled NRG-4, at 100-fold molar excess, together with the radioactive ligand and observed efficient displacement from IgB-4 (lower panel of Figure 4a). Thus, consistent with the ability of NRG-4 to induce growth and survival of ErbB-4-expressing cells, but not cells singly expressing the other three ErbBs, this ligand recognized only ErbB-4 (IgB4) in solution.

To test the prediction that NRG-4 can recognize a surface-expressed ErbB-4, but no other membrane-bound ErbB protein, we used a Chinese hamster ovary (CHO) cell line. These cells express low amounts of ErbB-2, but no other ErbB receptor, and accordingly did not bind NRG-4 or any other neuregulin (Tzahar *et al.* (1996), and data not shown). CHO cells were transfected with plasmid vectors directing expression of

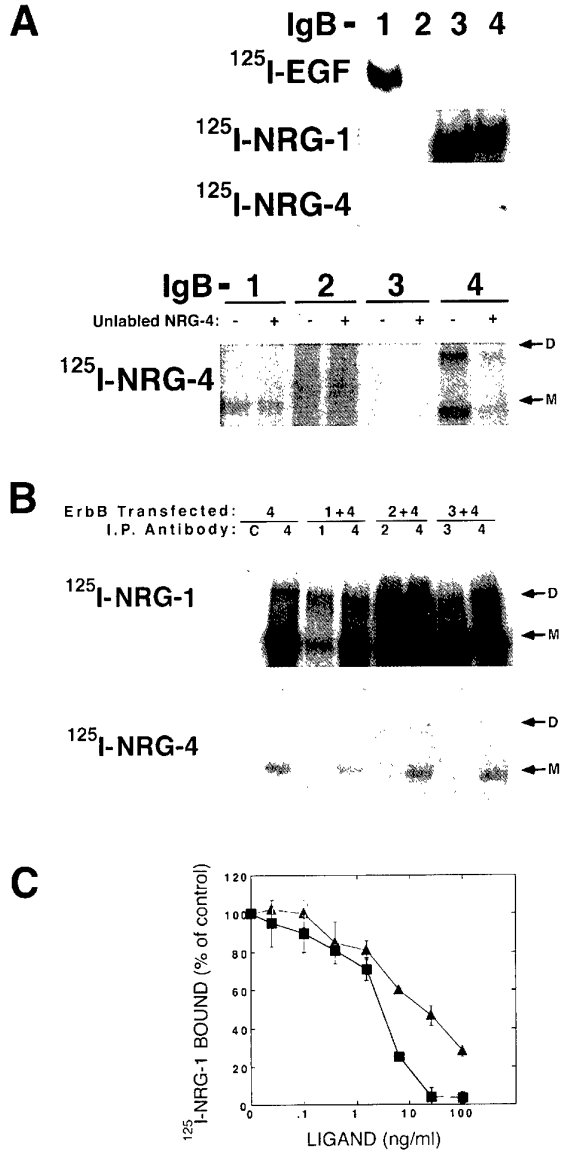

Figure 4 NRG-4 binding to ErbB proteins. (**a**) Covalent crosslinking of NRG-4 to recombinant-soluble ErbB proteins. The four soluble forms of ErbB proteins, in the form of IgG fusions (denoted IgB-1 through -4), were separately incubated with the indicated radiolabeled growth factors. Where indicated (lower panel), an excess (100-fold) of unlabeled NRG-4 was co-incubated with the labeled ligand. Following 2 h at 22°C, the covalent crosslinking reagent BS^3 was added (1 mM) and 45 min later the ligand-receptor complexes were immunoprecipitated with agarose-immobilized protein-A beads. Arrows mark the locations of monomeric (M) and dimeric (D) receptor complexes. (**b**) Covalent crosslinking of NRG-4 to cell surface-expressed ErbB proteins. CHO cells were transfected with vectors directing expression of the indicated ErbB proteins or their combinations. Two days later cell monolayers were incubated with either ^{125}I-NRG-1β or ^{125}I-NRG-4 (EGF-like domains, each at 100 ng/ml). Following 2 h at 4°C, the covalent crosslinking reagent *bis*(sulfosuccinimdyl)-suberate (BS^3) was added (1 mM final concentration) and cell extracts prepared after an additional 45 min of incubation. The indicated ErbB proteins were then immunoprecipitated (I.P.) with mouse monoclonal antibodies, and the complexes resolved by gel electrophoresis and autoradiography. Arrows mark the locations of monomeric (M) and dimeric (D) receptor complexes. (**c**) Binding of NRG-4 to ErbB-4-expressing cells. Displacement analyses of radiolabeled NRG-1β were performed with CHO cells expressing ErbB-4. Cell monolayers (2×10^5 cells) were incubated for 2 h at 4°C with a radiolabeled NRG-1 (5 ng/ml) in the presence of increasing concentrations of an unlabeled NRG-4 (closed triangles), or NRG-1β (closed squares). Each data point represents the mean and range (bars) of two determinations

ErbB-4, or co-transfected with an ErbB-4 plasmid together with vectors expressing one of the three other ErbB proteins. Two days later, cells were incubated with ^{125}I-NRG-4, or with a radiolabeled NRG-1 as control, and subsequently the formed ligand-receptor complexes were stabilized by using a covalent cross-linking reagent. Immunoprecipitation of the expressed ErbB proteins allowed analysis of the covalently held complexes. Expression of ErbB-4 alone conferred to CHO cells the ability to form complexes with NRG-4, as well as with NRG-1 (Figure 4b, and data not shown). In line with the lower mitogenic activity of NRG-4, the covalent crosslinking signal obtained with this ligand was weaker than that observed with a radioactive NRG-1. Nevertheless, both monomers and dimers of ErbB-4 were formed by the two ligands (detection of NRG-4-containing dimers required long film exposures). Co-expression of ErbB-1 or ErbB-3 did not significantly affect the radioactive signals, but in the case of ErbB-2 an enhancement effect was observed with NRG-1. The ability of anti-ErbB-1 and anti-ErbB-2 antibodies to precipitate NRG-4-labeled monomeric and dimeric receptor species (Figure 4b) is probably due to co-immunoprecipitation of ErbB-4 and it indicates the existence of NRG-4-promoted heterodimers with ErbB-1 and ErbB-2. Interestingly, ErbB-3 largely escaped heterodimerization with ErbB-4 following binding of NRG-1 or NRG-4.

Taken together, the biological effects of NRG-4 and its complex formation with ErbB-4 implied not only specificity of recognition, but also weaker interaction relative to NRG-1. To quantify the interaction, we performed ligand displacement analysis on ErbB-4-expressing CHO cells. The ability of unlabeled NRG-4 to displace surface-bound radiolabeled NRG-1-β was compared with that of unlabeled NRG-1. The results of this experiment indicated an approximately eightfold lower binding affinity of NRG-4 to ErbB-4 (Figure 4c). While NRG-1 bound with an apparent affinity that lies in the low nM range, NRG-4 displayed an apparent approximate Kd of 20 nM. In conclusion, NRG-4 specifically binds to ErbB-4 with an affinity that is lower than that of NRG-1β. Because we previously reported that relative to NRG-1β the alpha isoform displays a 5–8-fold lower affinity to both ErbB-3 and ErbB-4 (Tzahar *et al.*, 1994), it is conceivable that NRG-4 and NRG-1α bind to ErbB-4 with similar affinities.

Evidently, NRG-4 binds to ErbB-4 and mediates cell proliferation through activation of this receptor. Because other ErbB ligands stimulate cell growth via tyrosine phosphorylation of their respective receptors and activation of the intervening mitogen-activated protein kinase (MAPK) cascade, we tested these two signaling steps in NRG-4-responsive myeloid cells expressing ErbB-4 (D4 and D24 cell lines). Cells were stimulated with 100 ng/ml of activating ligand for 5 min, followed by lysis and analysis by immunoblotting. NRG-4 stimulated phosphorylation of the 180-kDa ErbB receptors in D4 and in D24 cells with an

accompanying activation of MAP-kinase (Erk-1 and Erk-2) also detected (Figure 5). In contrast to these two cell lines, and consistent with the growth and binding assays, NRG-4 at doses as high as 1 μg/ml, did not stimulate the other 32D cell lines (D1, D2, D3, D12, D13 and D23 cells, data not shown). These results further support the conclusion that NRG-4 is a bona fide ligand of the ErbB receptor family that selectively interacts with receptor complexes containing ErbB-4.

Discussion

In this paper we identify and present the initial characterization of NRG-4, a new cognate ligand of the EGF/NRG family. Aside from NRG-4 possessing a neuregulin-like EGF domain (Figure 1c), it shares very little other sequence homology to the known NRGs, particularly in the vicinity of the transmembrane domain, a region where the other three NRGs exhibit high primary sequence homology. However, the presumed precursor form of NRG-4 shares several structural characteristics with other mammalian ErbB ligands (reviewed in Massague and Pandiella (1993)), including a transmembrane topology, a juxtamembrane location of the EGF-like domain, and a putative proteolytic cleavage site located at a serine-rich region C-terminally to the EGF-like domain. This region may serve as a site of O-glycosylation, in addition to two potential sites of N-glycosylation located in the presumed ectodomain of NRG-4. Like other NRGs, but unlike most ErbB-1-specific ligands, NRG-4 lacks an N-terminally located hydrophobic signal peptide. However, the absence of a characteristic sequence may not exclude the possibility that NRG-4 acts as a secreted growth factor, because other signal peptide-less growth factors can be secreted or released from producer cells through alternative secretory mechanisms or upon cell lysis. NRG-4 presents a rather unique case as it also lacks an apolar stretch of amino acids that usually replaces a signal peptide (e.g., in NRG-1).

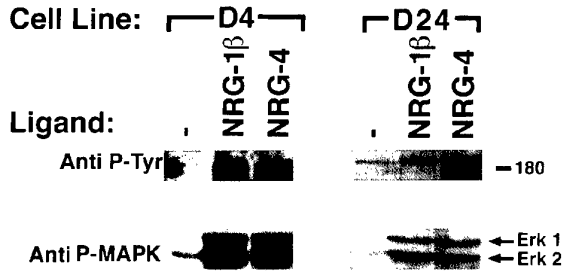

Figure 5 Tyrosine phosphorylation and MAPK activation by NRG-4. Derivatives of 32D cells expressing ErbB-4, either alone (D4 cells) or in combination with ErbB-2 (D24 cells) were incubated for 5 min at 37°C with either NRG-4 or NRG-1β (each at 100 ng/ml). Whole cell extracts were then prepared, resolved by gel electrophoresis, and transferred to a nitrocellulose filter. The upper portion of the filter was immunoblotted with antibodies to phosphotyrosine (P-Tyr, the 150–200 kDa region is shown) or an antibody directed at the activated doubly phosphorylated form of the MAPK (Erk1 and Erk2, the 40–50 kDa region is shown). Antibodies were incubated with a secondary reagent coupled to horseradish peroxidase, allowing subsequent detection by chemiluminescence

In fact, the presumed ectodomain of NRG-4 is the shortest among NRG/EGF family members. In addition, unlike other NRGs, which contain a variety of structural motifs, such as an immunoglobulin-like domain, a cysteine-rich region, or a mucin-like domain, NRG-4 contains no recognizable structural motif other than the EGF-like domain.

That the EGF-like domain of NRG-4 functions as a receptor-binding moiety is indicated by our *in vitro* studies with engineered cell lines and also with breast cancer cells naturally expressing all four ErbB proteins (Figures 3–5, and data not shown). The EGF-like domain of NRG-4 exhibits restricted binding specificity, it directly binds to ErbB-4, but not to ErbB-1, ErbB-2 or ErbB-3. A similar selective binding to ErbB-4 has also been reported for NRG-3 (Zhang *et al.*, 1997) and may indicate that during development and in the adult, ligands with restricted ErbB specificities may play important roles. It is interesting to note that NRG-3 is the EGF-like ligand closest to NRG-4 (42% sequence identity in the EGF-like domain). Also relevant is the emerging wider repertoire of ErbB-4-specific ligands, as compared with growth factors that bind to ErbB-3. In addition to NRG-1, NRG-2, and NRG-3, ErbB-4 also binds to betacellulin (Riese *et al.*, 1996a), epiregulin (Shelly *et al.*, 1998) and HB-EGF (Elenius *et al.*, 1997). Moreover, at high ligand concentrations, or in the presence of a co-expressed ErbB-2, ErbB-4 also binds EGF and TGFα (Shelly *et al.*, 1998; Wang *et al.*, 1998). The broader specificity of ErbB-4 was reflected also in mutagenesis experiments: more NRG-1 mutants displayed greater affinity loss for ErbB-3 compared with ErbB-4 (Jones *et al.*, 1998).

Besides specificity to ErbB-4, NRG-3 and NRG-4 share relatively low affinity to this receptor compared with NRG-1 (Figure 4 and Zhang *et al.*, 1997). Several other ligands, such as epiregulin (Shelly *et al.*, 1998) and the alpha isoform of NRG-1 (Tzahar *et al.*, 1994), also display relatively low affinity to ErbB-4. These observations may suggest the existence of additional, yet undiscovered ErbB proteins, serving as high affinity receptors for these low affinity ligands. Alternatively, low affinity ligands may have a different biological function than high affinity growth factors, as they can escape the common rapid endocytic clearance from the extracellular space (Reddy *et al.*, 1996; Shelly *et al.*, 1998). Alternatively, the ligand-less co-receptor of ErbB-4, namely ErbB-2 (Karunagaran *et al.*, 1996), may be more effective in the case of low affinity ligands, such as NRG-3 and NRG-4, thus offering a mechanism for fine-tuning of ErbB signaling. The interaction of ErbB ligands with ErbB-2 appears to involve direct binding to an ErbB-2 promiscuous binding site (Klapper *et al.*, 1997; Tzahar *et al.*, 1997). According to this model, all EGF-like growth factors are bivalent ligands, that differ in their binding specificity to specific pairs of ErbB receptors (Tzahar *et al.*, 1997). This hypothesis may explain the multiplicity of ErbB ligands in terms of their differential ability to stabilize homo- and heterodimeric ErbB proteins. When applied to NRG-4, the bivalence model predicts that this ligand may differ from other ErbB-4-specific ligands, including NRG-3, in the ability to recruit heterodimer partners to ErbB-4.

Consistent with this model, we demonstrate that when co-expressed with ErbB-4, NRG-4 can recruit

both ErbB-1 and ErbB-2 into heterodimers (Figure 4b). These NRG-4-induced heterodimeric complexes may be of physiological importance, as indicated in proliferation assays: NRG-4 weakly stimulated the growth of myeloid cells engineered to express ErbB-4 alone (D4 cells). In contrast, this response was significantly enhanced upon ErbB-2 co-expression (D24 cells, Figure 3) when compared to that of the internal NRG-1 control. This finding may indicate that under some physiological conditions, the expression of ErbB-4 alone may be insufficient to elicit a biological response to NRG-4, requiring a co-receptor such as ErbB-2 to transduce its signal. This scenario has a precedence in the case of NRG-1: *in vitro* experiments showed clear enhancement of an ErbB-4-mediated mitogenic effect by a co-expressed ErbB-2 (Wang *et al.*, 1998), and gene-targeting in mice indicated that ErbB-2 is essential for cardiac trabeculation that is mediated by NRG-1 and ErbB-4 (Lee *et al.*, 1995).

With the exception of EGF, which is found in high concentrations in body fluids such as milk, urine and saliva (Carpenter and Cohen, 1979; Gregory *et al.*, 1979), all of the EGF/NRG family members are thought to act as short-range ligands affecting only neighboring cells through paracrine or autocrine loops (reviewed in Ben-Baruch *et al.*, 1998). Consistent with short-range ligand-receptor interactions, NRG-3 is expressed primarily in the central nervous system, along with its only known receptor, ErbB-4 (Plowman *et al.*, 1993; Zhang *et al.*, 1997). However, ErbB-4 is expressed also in muscle, heart, pancreas, salivary gland and lung (Gassmann *et al.*, 1995; Pinkas-Kramarski *et al.*, 1997; Plowman *et al.*, 1993). Our Northern blot analysis (Figure 2) demonstrated that in the adult, two of these ErbB-4-positive tissues, pancreas and muscle, express three molecular weight species of NRG-4. Likewise, multiple mRNA species of NRG-1 and NRG-2 were reported (Chan *et al.*, 1995; Wen *et al.*, 1992). Whether or not the multiplicity of NRG-4 mRNAs is related to the existence of many isoforms of NRG-1 and NRG-2 (Busfield *et al.*, 1997; Carraway *et al.*, 1997; Chang *et al.*, 1997; Marchionni *et al.*, 1993; Wen *et al.*, 1994) is currently unknown.

In summary we describe here the first characterization of NRG-4, a novel member of the ErbB ligand family, whose structure, expression pattern and restrained receptor-binding properties suggest a unique physiological role. Gene-targeting and *in vitro* studies with recombinant NRG-4 may resolve the presumed distinct biological role of this growth factor and its relationship to other EGF/NRG family ligands.

Materials and methods

Materials

EGF (human, recombinant) was purchased from Boehringer Mannheim. Recombinant human NDF$\beta 1_{177\ 246}$ (NRG1-β1) was obtained from Amgen (Thousand Oaks, CA, USA). Iodogen and bis(sulfosuccinimidyl) suberate (BS^3) were from Pierce. Monoclonal antibodies (mAbs) to ErbB proteins (Chen *et al.*, 1996; Klapper *et al.*, 1997) were used for immunoprecipitation. The composition of buffered solutions was described (Tzahar *et al.*, 1994). Recombinant soluble extracellular domains of the four ErbB proteins (denoted IgB-1 through -4) (Chen *et al.*, 1996), in the form of fusion proteins containing the Fc portion of human immunoglobulin G (IgG) were harvested from serum-free conditioned media of transfected HEK-293 human embryonic kidney cells. The PY20 antibody was purchased from Santa Cruz Biotechnology. A mAb to the active form of the MAP kinase (Yung *et al.*, 1997) was a gift from R Seger (Weizmann Institute).

Peptide synthesis

The EGF-like domain of NRG-4 (residues 4–50) was synthesized on an Applied Biosystems (ABI) 430A peptide synthesizer using standard *tert*-butyloxycarbonyl (*t*-Boc) chemistry protocols as described (Barbacci *et al.*, 1995). Acetic anhydride capping was employed after each activated ester coupling. The peptide was assembled on phenylacetamidomethyl polystyrene resin using standard side chain protection, except for the use of *t*-Boc-Glu(O-cyclohexyl) and *t*-Boc-Asp(O-cyclohexyl). The peptide was deprotected using the 'Low-High' hydrofluoric acid (HF) method (Tam *et al.*, 1983). The crude HF product was purified by reverse phase HPLC (C-18 Vydac, 22 × 250 mm), diluted without drying into folding buffer (1 M urea, 100 mM Tris, pH 8.0, 1.5 mM oxidized glutathione, 0.75 mM reduced glutathione, 10 mM methionine), and stirred for 48 h at 4°C. The folded, fully oxidized peptide was purified from the folding mixture by reverse phase HPLC, and characterized by electrospray mass spectroscopy. A single HPLC peak with an averaged molecular mass (Mr) of 5371.50 was displayed by the reduced peptide prior to folding. This mass is in agreement with the theoretical Mr (5371.20). The folded and oxidized peptide displayed a slightly lower averaged molecular mass of 5366.88.

Database searches

EST databases were scanned for homology to the EGF-like domain of NRG-1β (NDF-β) by Blast and Smith-Waterman algorithms (Samuel and Altschul, 1990; Smith and Waterman, 1981) using both a Unix-interfaced GCG server and a Bioaccelerator device (Compugen, Israel).

Northern blot

A Northern blot filter was purchased from Clontech (MTN Blot #7760-1), each lane containing approximately 2 μg of poly(A)$^+$ purified mRNA from healthy human tissues and run on a denaturing 1.2% formaldehyde/agarose gel. Hybridization to cDNA probes to mouse *NRG-4* and human β-actin were performed with 'ExpressHyb' (Clontech) using the protocol provided by the manufacturer. Probing with a human amylase cDNA probe was performed by standard techniques. After each hybridization, blots were washed at room temperature for 40 min with several changes of low stringency wash solution (2 × SSC, 0.05% SDS) and then with at least two changes of high stringency buffer (0.1 × SSC, 0.1% SDS) at 50°C for 40 min.

Lysate preparation for Western blot analyses

For receptor activation studies, derivatives of the 32D cell line were resuspended in phosphate-buffered solution (PBS) and incubated at 22°C for 15 min before adding growth factors and incubating for 5 min at 37°C. Cells were then pelleted and lysed in ice cold solubilization buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Noidet-P-40, 0.5% Na-deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1.5 mM EDTA, 1.5 mM $MgCl_2$, 2 mM Na-orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml aprotinin and 10 μg/ml leupeptin) and left on ice for 15 min. The whole cell extract was then cleared by centrifugation (12 000 *g* for 10 min at 4°C), immediately boiled in reducing gel sample buffer, and resolved by 10% SDS–PAGE before being

transferred onto nitrocellulose. Filters were blocked in TBST buffer (0.02 M Tris-HCl [7.5], 0.15 M NaCl, 0.05% Tween-20) containing 1% milk for 40 min at 22°C, blotted with primary antibodies in TBST overnight at 4°C, followed by conjugation with a secondary antibody linked to horseradish peroxidase and subsequent detection by chemiluminescence (Amersham Corp.).

Radiolabeling of ligands, covalent crosslinking and ligand displacement analyses

Growth factors were labeled with Iodogen (Pierce) as described (Karunagaran *et al*., 1995). Chemical crosslinking to Chinese hamster ovary (CHO) cells engineered to express different ErbB combinations have been performed essentially as described (Tzahar *et al*., 1996). Briefly, radiolabeled ligands (at 100 ng/ml) were incubated for 2 h with cell monolayers at 4°C. The chemical crosslinking agent BS^3 (1 mM) was then added and the cells were further incubated for 45 min at 22°C. Mouse antibodies were first coupled to rabbit anti-mouse IgG and to protein A-Sepharose beads, and then they were incubated with cell extracts for 2 h at 4°C. Immunoprecipitated complexes were then washed three times with ice-cold SBN buffer (1% NP-40; 150 mM NaCl; 10% Glycerol; 1 mM EGTA, in 50 mM Tris-HCl, pH 7.4; 1 ml per wash) prior to heating (5 min at 95°C) in gel sample buffer, resolution by gel electrophoresis, transfer to nitrocellulose and autoradiography. For crosslinking with IgBs, after co-incubation of IgB-containing conditioned media with radiolabeled ligands, complexes were immunoprecipitated directly with Sepharose-protein A beads. For ligand displacement analyses, cell monolayers were washed once with binding buffer, and then incubated for 2 h at 4°C with radiolabeled NRG-1β (5 ng/ml) and various concentrations of unlabeled ligands, as indicated. Non-specific binding was determined in the presence of a 100-fold molar excess of the unlabeled ligand. Cells were then washed, lysed in a solution containing 0.1 M NaOH and 0.1% SDS, and radioactivity determined by use of a gamma counter.

Cell proliferation assays

The establishment of a series of interleukin 3- (IL-3-) dependent 32D myeloid cells expressing all combinations of ErbB proteins has been described (Alimandi *et al*., 1997; Pinkas-Kramarski *et al*., 1996; Shelly *et al*., 1998). Cells were maintained in RPMI medium with 10% fetal bovine serum (FBS) and dilute IL3-containing conditioned medium. Prior to proliferation assays, cells were washed three times in RPMI/FBS and plated (5×10^5 cells/ml; 0.1 ml/well) into 96-well flat-bottomed plates with the indicated ligand concentrations or with IL-3 (1:1000 dilution of conditioned medium). Cell survival was determined 24 h later, or after the indicated time intervals, by MTT assay, as previously described (Mosman, 1983). MTT (0.05 mg/ml) was incubated with the analysed cells for 2 h at 37°C. Living cells can transform the tetrazolium ring into dark blue formazan crystals, that can be quantified by reading the optical density at 540–630 nm after lysis of the cells with acidic isopropanol.

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Cell Biology

The ErbB-2/HER2 oncoprotein of human carcinomas may function solely as a shared coreceptor for multiple stroma-derived growth factors

LEAH N. KLAPPER*†, STEFANIE GLATHE†‡, NORA VAISMAN*†, NANCY E. HYNES§, GLENN C. ANDREWS¶, MICHAEL SELA*, AND YOSEF YARDEN†‖

Departments of *Immunology and †Biological Regulation, Weizmann Institute of Science, Rehovot 76100, Israel; §Friedrich Miescher Institute, CH-4002 Basel, Switzerland; and ¶Pfizer Central Research, Groton, CT 06340

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ABSTRACT **The *erbB-2/HER2* oncogene is overexpressed in a significant fraction of human carcinomas of the breast, ovary, and lung in a manner that correlates with poor prognosis. Although the encoded protein resembles several receptors for growth factors, no high affinity ligand of ErbB-2 has so far been fully characterized. However, several lines of evidence have raised the possibility that ErbB-2 can augment signal transduction initiated by binding of certain growth factors to their direct receptors. Here, we contrasted these two models of ErbB-2 function: First, examination of a large series of epidermal growth factor (EGF)-like ligands and neuregulins, including virus-encoded ligands as well as related motifs derived from the precursor of EGF, failed to detect interactions with ErbB-2 when this protein was singly expressed. Second, by using antibodies that block inter-ErbB interactions and cells devoid of surface ErbB-2, we learned that signaling by all ligands examined, except those derived from the precursor of EGF, was enhanced by the oncoprotein. These results imply that ErbB-2 evolved as a shared receptor subunit of all ErbB-specific growth factors. Thus, oncogenicity of ErbB-2 in human epithelia may not rely on the existence of a specific ligand but rather on its ability to act as a coreceptor for multiple stroma-derived growth factors.**

Cellular growth and fate determination are controlled by a large variety of extracellular ligands and specific cell surface receptors. The largest family of such receptors is that of the growth factor receptors with intrinsic tyrosine kinase activity (1). Type-1 tyrosine kinase receptors, also known as ErbB/HER proteins, comprise one of the better-characterized subfamilies of growth factor receptors, of which the epidermal growth factor (EGF) receptor (ErbB-1) is the prototype (reviewed in ref. 2). The four ErbB members form homo- and heterodimeric complexes on binding of EGF-like or neuregulin (NRG) ligands, and, thereby, their kinase activity is stimulated and intracellular signals are generated. Constitutive stimulation of these pathways through autocrine or other mechanisms is associated with several types of human cancer (3). Most relevant is the frequent overexpression, often as a result of gene amplification, of ErbB-2/HER2 in breast, ovary, lung, and other types of epithelial cancers (reviewed in refs. 4 and 5). In some tissues, this overexpression was correlated with poorer prognosis and a more aggressive tumor phenotype (6).

Although ErbB-2 shares extensive structural homology with other ErbBs both along the catalytic intracellular domain and in the extracellular putative ligand binding region, many attempts to identify stimulatory ligands specific to ErbB-2 have so far failed. For example, detection of an activity that enhances ErbB-2 phosphorylation led to molecular cloning of the Neu differentiation factor (NDF) and heregulin, two of a dozen isoforms of NRG1, all of which bind to ErbB-3 and ErbB-4 (7). Nevertheless, several observations imply that ErbB-2 homodimers, the plausible outcome of a direct ligand, may be functional *in vivo*. An oncogenic mutation that activates ErbB-2 phosphorylation apparently stabilizes such homodimers (8), and bivalent anti-ErbB-2 antibodies are mitogenic because they, like a direct ligand, dimerize ErbB-2 on the cell surface (9).

In parallel with attempts to isolate a direct ligand, several approaches culminated at the possibility that ErbB-2 functions, at least in part, as a coreceptor. Thus, coexpression of ErbB-2 together with ErbB-1 enhanced EGF-induced mitogenesis (10), and ErbB-2 presence reconstituted an extremely potent proliferative activity of ErbB-3, which is totally inactive when singly expressed (9). Consistent with its transactivating capability, ErbB-2 was found to act as the preferred partner of ligand-driven ErbB heterodimers (11, 12). The use of intracellular antibodies to ErbB-2 (13) has led to the conclusion that it can enhance signaling by two growth factors, EGF and NDF, through an ability to decelerate their release from the direct receptors, namely ErbB-1 and either ErbB-3 or ErbB-4, respectively (14).

Does ErbB-2 function as a high affinity receptor for a still unknown ligand of the EGF/NRG families, or could it act solely as a shared receptor subunit that amplifies signaling by prolonging the action of heterologous ligands? The present study addressed this question by using two strategies: First, we examined ligands that have not been previously tested for direct interaction with ErbB-2. On the other hand, we analyzed the generality of the transactivation ability of ErbB-2 by combining most existing ErbB ligands with mAbs that block a putative ligand binding site of ErbB-2. Our results strongly support the possibility that ErbB-2 evolved as a pan-EGF/NRG receptor rather than a high affinity receptor for a novel ligand. The implications of this scenario to epithelial tumors overexpressing ErbB-2 and to their inductive interactions with the underlying mesenchyme are discussed.

MATERIALS AND METHODS

Materials, Cell Lines, and Antibodies. The construction and sources of recombinant and synthetic growth factors were as previously specified (15, 16, 17). Recombinant soluble extra-

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Abbreviations: EGF, epidermal growth factor; NDF, Neu differentiation factor; NRG, neuregulin; SFGF, Shope fibroma virus growth factor; proEGF, precursor of the epidermal growth factor; GST, glutathione *S*-transferase; MAPK, mitogen-activated protein kinase; IgB, ErbB extracellular domains fused to an Fc portion of human Ig G.

‡Present address: Procter and Gamble, Edificio Alvares Carbal, Quinta da Fonte, Porto Salvo 2780, Oeiras, Portugal.
‖To whom reprint requests should be addressed. e-mail: liyarden@weizmann.weizmann.ac.il.

cellular domains of ErbB proteins fused to the Fc portion of human immmunoglobulin G (IgB) have been described (18). Antibodies directed against ErbB-2, used for receptor activation and immunoprecipitation, have been described (19), as have those against ErbB-3 and ErbB-4 (18). An antiphosphotyrosine mAb (PY-20) was purchased from Santa Cruz Biotechnology. A mAb to the active form of the mitogen-activated protein kinase (MAPK) (20) was a gift from R. Seger (Weizmann Institute). T47D human breast cancer cells and their derivative T47D-5R have been described (13). 32D myeloid cells that ectopically express ErbB receptors have been described (9).

Expression of Recombinant Precursor of EGF (proEGF) Fusion Proteins. Four fragments containing the EGF-like domains of proEGF were constructed by PCR reactions on the full-length cDNA sequence of human proEGF in the pHEGF502 vector (kindly provided by Graeme I. Bell, Howard Hughes Medical Institute, Chicago) (21). The fragments, denoted pro1–4 (amino acids 314–479), pro5–8 (amino acids 741–952), pro5–9 (amino acids 741-1023), and EGF (amino acids 970-1023) were inserted into the pGEX expression vector (Amersham Pharmacia). Bacteria transformed with the constructs were induced to express the proteins and were harvested and lysed. Centrifugation-cleared lysates were mixed with glutathione-agarose beads and were incubated at 4°C while gently shaking. Elution of the bound proteins was carried out with 15 mM reduced glutathione and was followed by dialysis against PBS.

Cell Lysate Preparation. Cells grown as monolayers were solubilized as described (19). Proteins were separated electrophoretically either directly or after immunoprecipitation, were transferred to a nitrocellulose membrane, and were detected by immunoblotting.

Determination of Tyrosine Phosphorylation and MAPK Activation. Cells were incubated in PBS containing various ligands or mAbs at 37°C for the indicated time intervals. The treatment was ended by washing with ice-cold PBS. Whole cell lysates or immunoprecipitates were immunoblotted with an antiphosphotyrosine antibody (PY-20) or with a mAb that recognizes the doubly phosphorylated form of MAPK (20).

Cell Proliferation Assays. Proliferation of IL-3-dependent 32D cells expressing ErbB proteins was determined as described (9).

RESULTS

ErbB Ligands Cannot Activate a Singly Expressed ErbB-2, but Multiple Growth factors Can Activate it in Epithelial Cancer Cells. The ability of ErbB-2 to serve as a surrogate receptor when coexpressed with other family members, as well as the so-far unsuccessful search for a specific ErbB-2-binding ligand, suggest that its importance may reside in an intrinsic capacity to enhance signaling by a vast majority of ErbB-stimulating ligands. To experimentally test this scenario, we used an engineered 32D myeloid cell line that originally expresses no ErbB protein (9) and a large variety of known ErbB ligands (either EGF-like or NRGs). 32D cells that singly express ErbB-2 (D2) were incubated with growth factors, and the stimulation of ErbB-2 was followed by examining its phosphorylation on tyrosine residues (Fig. 1*A*). None of the 10 ligands tested was able to stimulate ErbB-2. That the protein is stimulatable under these conditions was evident from the ability of a mAb to ErbB-2 [L140 (19)] to stimulate tyrosine autophosphorylation. Antibody bivalency is essential for kinase stimulation (19), indicating that homodimerization of ErbB-2, a bona fide attribute of a direct ErbB-2 ligand, is functional in D2 cells. By contrast with their inability to stimulate a singly expressed ErbB-2, all 10 ligands we examined stimulated ErbB-2 phosphorylation to different extents in SKOV-3 ovarian cancer cells (Fig. 1*B*), which express ErbB-2

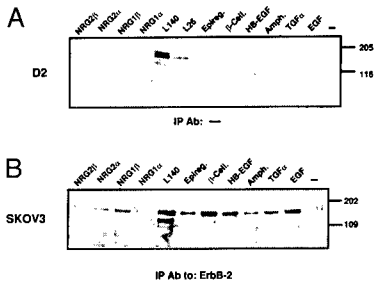


FIG. 1. ErbB-2 activation depends on coexpression of other ErbB proteins. ErbB-2 phosphorylation was determined in cells expressing the receptor singly (*A*, D2) or in combination with ErbB-1 and ErbB-3 (*B*, SKOV3). The indicated ligands (100 ng/ml) or antibodies (20 μg/ml) were used to treat the cells for 5 min at 37°C. Receptor activation in whole cell lysates (*A*) or immunoprecipitates of ErbB-2 (*B*) was determined by an antibody directed against phosphorylated tyrosine.

along with ErbB-1 and ErbB-3. To exclude dependence on cell type, we also examined Chinese hamster ovary cells, which express ErbB-2 in the absence of other ErbB members, and T47D breast cancer cells that express all four ErbBs. Similar to the results presented in Fig. 1, none of the growth factors tested was able to activate ErbB-2 in the former, but all ligands were active on the latter cell type (data not shown). In conclusion, although homodimeric stimulation of ErbB-2 is achievable, its activation by hitherto identified ErbB ligands strictly depends on coexpression of other receptor partners.

ErbB-2 Augments Stimulation of Mitogenesis by Multiple ErbB Ligands. Because ErbB-2 can enhance signaling by NDF and EGF (14) and it is the preferred heterodimerizing partner of the respective receptors (11, 12), we hypothesized a similar role for this receptor in the transmission of signals by the majority of ErbB ligands. To examine the involvement of ErbB-2 in signaling by additional ligands, we applied mAbs that can inhibit ErbB-2 interactions with its family members [class II mAbs (19)] and 32D myeloid cells expressing defined ErbB combinations (9, 15). When deprived of IL-3, these cells totally depend on exogenous growth factors for survival. Cells expressing ErbB-2 with either ErbB-1 (D12), ErbB-3 (D23), or ErbB-4 (D24) were stimulated by EGF-like ligands in the presence of ErbB-specific mAbs. NRGs of several isoforms (NRG1α, NRG1β, and NRG2α) induced cellular proliferation by promoting complexes containing ErbB-2 in combination with either ErbB-3 or ErbB-4 (Fig. 2; data not shown). This effect could be significantly decreased by anti-ErbB-2 antibodies capable of heterodimer destabilization (L26 and L96), as well as by their monovalent fragments (F26). mAbs directed against different epitopes (L87, L140, and L431) were incapable of exerting a similar effect, suggesting that interreceptor interactions, stimulated by all of the examined ligands, depend on a similar domain of ErbB-2. Inhibition of mitogenicity stimulated in cells coexpressing ErbB-2 with ErbB-3 was marked and similar in extent to that achieved by a ligand-displacing antibody directed against ErbB-3 [mAb C105 (18)]. Mitogenic stimulation by ligands that primarily stimulate ErbB-1 exhibited a similar pattern of ErbB-2 dependency (Fig. 2, lower panels). As previously demonstrated for EGF (19), the L26 antibody inhibited proliferation induced by transforming growth factor α in D12 cells. Both betacellulin and epiregulin, which benefit from ErbB-2 participation in their signaling (15, 22), induced a decreased mitogenicity in the presence of mAb L26 in D23 and in D24 cells, respectively. Taken together, the results shown in Fig. 2 indicate that ErbB-2 is capable of increasing ligand-stimulated mitogenicity without discriminating between the heterodimerizing ErbBs and their respective ligands.

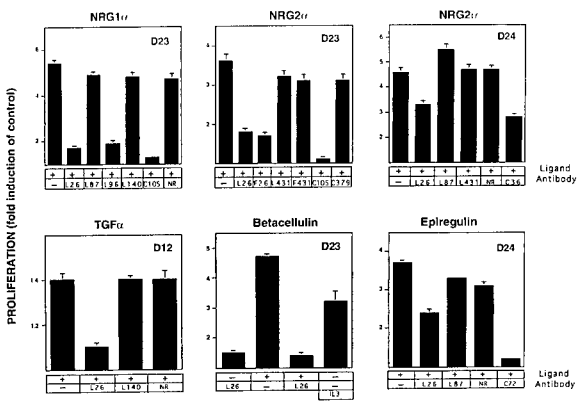


FIG. 2. ErbB-2-dependency of growth stimulation by EGF-like ligands. 32D cells expressing ErbB-2 with either ErbB-1 (D12), ErbB-3 (D23), or ErbB-4 (D24) were tested for cell proliferation. Cells deprived of IL-3 were treated with the indicated ligands. Anti-ErbB-2 mAbs belonging to class I (L431), class II (L26, L96), class III (L140), and class IV (L87) or their respective Fab fragments (F26, F431) were added simultaneously. Alternatively, control antibodies were used, including an unrelated mAb (NR), mAbs capable of ligand displacement from ErbB-3 (C105) or ErbB-4 (C72, C36), or an antibody against ErbB-3 that is incapable of displacing NRGs (C379). The extent of cell proliferation was determined 24 h after the addition of stimulating factors by using the colorimetric 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. The results are presented as fold induction over control untreated cells and are the mean ± SD of eight determinations. Note that most mAbs (e.g., L26) have a weak agonist activity of their own.

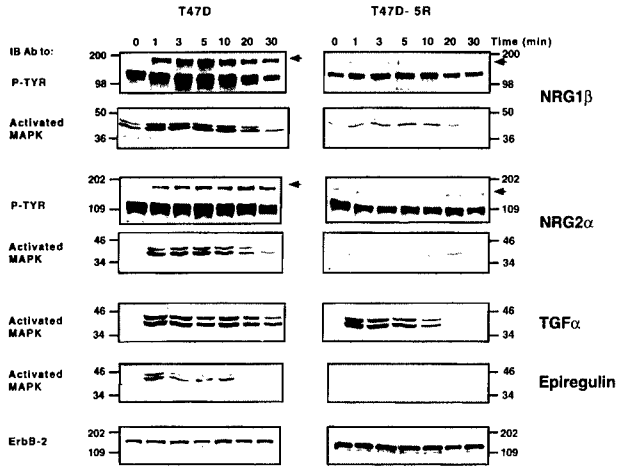


FIG. 3. The effect of surface-expressed ErbB-2 on the kinetics of ligand-induced tyrosine phosphorylation and MAPK activation. ErbB ligands were used to stimulate T47D breast cancer cells and their derivative, T47D-5R, which lacks surface expression of ErbB-2. A comparable number of cells was stimulated at 37°C by the indicated ligands (at 100 ng/ml) for various time intervals. Receptor activation, in whole cell lysates, was detected by immunoblotting (IB) with an antibody directed against phosphorylated tyrosine *(P-TYR)*. MAPK activation in the same preparations was determined by using an antibody against the active doubly phosphorylated form of Erk proteins (*Activated MAPK*). For control of equal gel loading, the upper part of membranes used to detect MAPK was used to determine the amount of ErbB-2. Note that the 5R cells exhibited up-regulation of the cell-retained ErbB-2.

ErbB-2 Enhances and Prolongs Signal Transduction by Multiple Growth Factors. Despite complexity of the ErbB signaling network, achieved by multiplicity of both ligands and receptors, signaling is funneled into a major cascade involving activation of the MAPK pathway. Recruitment of this pathway by an oncogenic ErbB-2 is essential for transformation (23), and ErbB-2 is known to augment signaling by EGF and NDF through MAPK (14). To pursue whether ErbB-2 involvement is a common cardinal element in signals promoted by ErbB ligands other than EGF and NDF, we used a breast cancer cell line, T47D, expressing all ErbB receptors and its derivative, T47D-5R, devoid of ErbB-2 surface expression due to intracellular entrapment (13). As demonstrated in Fig. 3, the parental cell line is induced, by different ligands, to activate the MAPK cascade, as determined by the detection of its two activated forms (20). Concomitant phosphorylation of a 180-kDa protein ensured the correlation between ErbB activation and subsequent events (shown for NRG1 and NRG2α). Comparing the kinetics of activation to that in cells lacking surface ErbB-2 revealed a significant inhibition of intracellular activation in the latter. Both receptor phosphorylation and MAPK activation were affected. Stimulation by NRGs was decreased in duration as well as in intensity in cells lacking surface ErbB-2. Likewise, transforming growth factor α, although capable of inducing a similar increase in MAPK phosphorylation to that in the parental cells, showed a significant reduction in activation kinetics in T47D-5R cells. Stimulation by an additional ErbB-1-activating ligand, epiregulin, was affected in a similar manner to that of NRGs, decreasing to a barely detectable level in the absence of surface ErbB-2. To validate adequate expression of ErbB receptors in the 5R derivative, their amount was compared with that in the parental strain (data not shown): ErbB-1, ErbB-3, and ErbB-4 exhibited unaltered expression in T47D-5R cells. ErbB-2, in these cells, showed a characteristic faster electrophoretic migration, confirming its retention in the endoplasmic reticulum (13). In conclusion, expression of ErbB-2 at the cell surface can significantly prolong signaling by several growth factors, suggesting a pan-ErbB stimulatory effect that is independent on ligand identity.

proEGF-Derived Units Are Unable to Recognize ErbB-2. Because the extracellular domain of ErbB-2 is homologous to the ligand-binding domains of other ErbB proteins and because all ErbB ligands share an EGF-like motif (24), an ErbB-2-specific ligand, if it exists, may include an EGF-like domain. Other possibilities, such as binding of a non-EGF-like ligand to a distinct site of ErbB-2, cannot, however, be excluded. The precursor of EGF, which shares transmembrane topology with most other precursors of ErbB ligands, includes nine EGF-like motifs, of which only the membrane proximal unit is an established growth factor (i.e., EGF). To examine whether other proEGF domains might harbor a capacity to recognize ErbB-2, we studied their functionality as separate fragments. Four recombinant fragments were designed: EGF-like domains I–IV (pro1–4), domains V–VIII (pro5–8), domains V–IX (pro5–9), and domain IX. The latter corresponds to the active unit, namely EGF, and served as a positive control. These protein fragments, as well as the analogous functional domain of NRG1α (NDF) were expressed in bacteria in the form of glutathione *S*-transferase (GST) fusion proteins. To ensure correct expression and folding of the putative ligands, the functional domains of both EGF (GST-EGF) and NDF (GST-NDF) were tested for binding *in vitro* to soluble ErbB receptors [IgBs (18)]. Binding of the soluble receptors, denoted IgB1 through IgB4, to glutathione agarose-immobilized ligands confirmed that both GST-EGF and GST-NDF retained their receptor specificity (Fig. 4*A Upper*). Examining domains of proEGF in a similar manner could not reveal any novel recognition (Fig. 4*A Lower*), although the recombinant proteins exhibited the correct molecular weights and reacted with antibodies directed to respective peptides (data not shown). That failure to detect interaction *in vitro* was not caused by protein misfolding was implied by the retention of IgB1 binding by the pro5–9 recombinant protein consisting of the functional domain IX (Fig. 4*A Lower*). The absence of this domain, as in the case of pro1–4 and pro5–8 proteins,

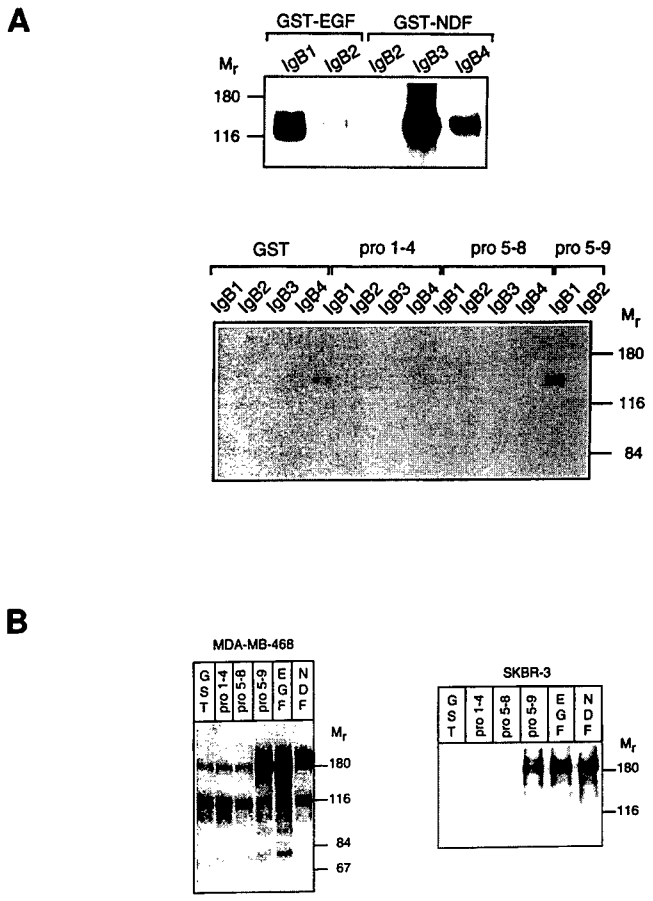


FIG. 4. Activation of ErbB receptors by EGF-like motifs of human proEGF. (*A*) GST fusion proteins containing EGF-like motifs 1–4, 5–8, or 5–9 of the EGF precursor were immobilized on glutathione-agarose beads. For control, GST fusion proteins containing EGF or NDF were used. The beads were incubated for 1 h at 4°C with conditioned media containing 1 μg of the indicated IgB protein. Protein complexes were immunoblotted with an anti-human Fc antiserum for detection of bound IgBs. (*B*) Monolayers of the indicated human breast cancer cell lines were incubated, for 10 min at 37°C, in the presence of 100 ng/ml GST fusion proteins or 5 ng/ml ligands (EGF or NDF). Receptor activation was detected by an antiphosphotyrosine antibody.

abolished recognition, reinforcing its sufficiency for receptor binding. Moreover, none of the fragments could recognize any other ErbB protein, although IgB3 and IgB4 bound NRGs, and IgB2 bound all tested mAbs to ErbB-2 (Fig. 4*A*; data not shown).

The inability of proEGF-derived units to act as ErbB-binding ligands was evident also from experiments performed with living breast cancer cells (Fig. 4*B*). By detecting phosphorylation of proteins on tyrosine residues in whole cell lysates, we could demonstrate a pattern of receptor activation which is in accordance with the above binding. Only fragments containing domain IX could activate phosphorylation of proteins corresponding to ErbB receptors. Moreover, comparing an ErbB-2 overexpressing cell line (SKBR-3) with one devoid of the receptor (MDA-MB-468) revealed a similar specificity of stimulation, namely the dependence of activation on the ninth EGF-like domain. Collectively, these results indicate that no other EGF-like domain derived from the precursor molecule could serve as an ErbB-2-specific ligand.

ErbB-2 Is Activated by Three Viral Ligands only when Coexpressed with Other Family Members. Three EGF-like ligands encoded by poxviruses have been shown to resemble ErbB-activating molecules in structure as well as in activity. These ligands, including the vaccinia virus growth factor, the

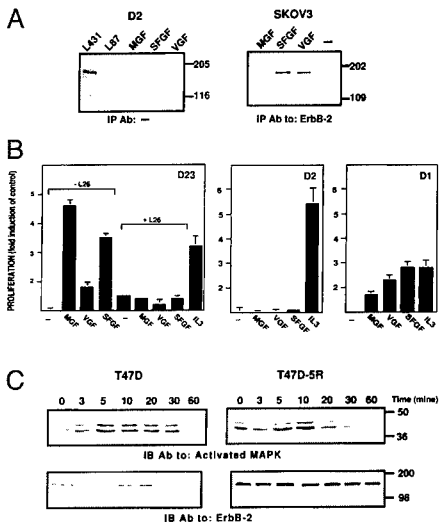


FIG. 5. Viral peptides recruit ErbB-2. (*A*) Phosphorylation of ErbB-2 by viral peptides [vaccinnia virus growth factor (VGF), Myxoma virus growth factor (MGF), and SFGF] and antibodies (L87, L431) was examined as described in the legend to Fig. 1. (*B*) IL-3-deprived D23 cells were stimulated by viral peptides in the presence *(+L26)* or absence *(−L26)* of a class II mAb to the human ErbB-2 (*Left*). Cells singly expressing ErbB-2 (D2) or ErbB-1 (D1) served as negative and positive controls for ligand activity, respectively. Proliferation induction was determined by the 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay as described in the legend to Fig. 2. For control of endogenous proliferation signals, we incubated cells with IL-3. (*C*) The effect of ErbB-2 on downstream activation by SFGF was examined in cells that do (T47D) or do not (T47D-5R) express ErbB-2 on their surface. A time response of activation was detected in whole cell lysates by immunoblotting with an antibody against activated MAPK. The amount of ErbB-2 was verified by immunoblotting the upper part of the membrane with an antibody against the receptor.

Shope fibroma virus growth factor (SFGF), and the Myxoma virus growth factor, harness the proliferation-inducing activity of ErbB receptors for the enhancement of their virulence (25). Synthetic analogs of these three viral ligands revealed specific patterns of ErbB specificity. For example, SFGF acts as a pan-ErbB ligand whereas Myxoma virus growth factor is more specific to the ErbB-2/ErbB-3 complex (17). Because evolutionarily the ErbB family evolved from a single protein whose ortholog in nematodes is Let-23 (26), and because it is likely that poxviruses coevolved with their vertebrate hosts (25), we assumed that an ErbB-2-specific ligand, if it ever existed, may have been retained in the genome of this large family of DNA viruses. To examine direct interaction between ErbB-2 and the three viral ligands, we used 32D cells singly expressing the protein (D2). As demonstrated in Fig. 5, none of the three known viral ligands promoted homodimerization of ErbB-2 and the consequent kinase activation (Fig. 5*A Left*) or mitogenic effect (Fig. 5*B Center*), although both activities were displayed by a mAb specific to ErbB-2 (Fig. 5*A*; data not shown).

Nevertheless, by using similar approaches to those presented above (Figs. 2 and 3), we learned that the three viral ligands, like their mammalian counterparts, depend on ErbB-2 for cellular activation. All three ligands could induce phosphorylation of ErbB-2 in SKOV3 cells (Fig. 5*A Right*), suggesting that the viral growth factors can recruit ErbB-2 into heterodimeric complexes. The involvement of ErbB-2 was also manifested biologically by a mitogenic assay (Fig. 5*B*); although none of the viral ligands was active on cells singly expressing the kinase-defective ErbB-3 receptor (data not shown), all three ligands potently stimulated cells coexpressing it with the ligand-less ErbB-2 (Fig. 5*B Left*). Recruitment of

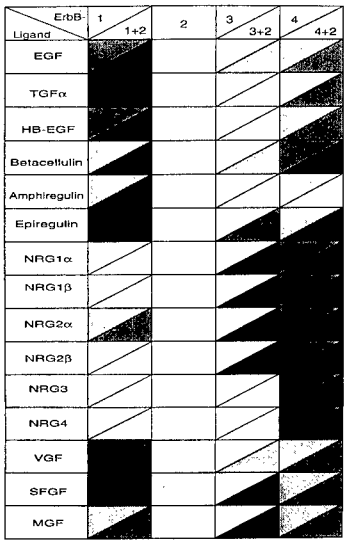
ErbB-2 by the viral ligands in these cells was evident also from the inhibitory activity of a class II mAb (L26) to ErbB-2 (Fig. 5*B*). Lastly, by using SFGF on T47D cells and the engineered 5R derivative, we observed an ErbB-2-mediated prolongation and enhancement of MAPK activation (Fig. 5*C*). Thus, although this ligand is capable of activating various ErbB complexes (17), it seems that SFGF, like the corresponding mammalian growth factors, depends on ErbB-2 as a coreceptor rather than as a direct high-affinity receptor.

DISCUSSION

Despite extensive investigation and a wealth of clinical data, the biochemical role of ErbB-2 in human cancer remains an enigma (4, 5): Although the structure and enzymatic function of the oncoprotein suggest that it is stimulated by a specific growth factor, *in vitro* studies along with the continuous failure to isolate a direct ligand imply a nonconventional receptor function (reviewed in ref. 27). This possibility has been strengthened by gene targeting experiments indicating cooperation between ErbB-2 and the neuregulin receptor ErbB-4 (28). By using a variety of ErbB ligands, our present study weakens the commonly held scenario arguing that ErbB-2 functions as an orphan receptor. Instead, a cooperative role in signal transduction is strongly supported.

The orphan receptor scenario predicts that an ErbB-2-specific ligand exists and that it contributes to tumor virulence by promoting homodimerization of the overexpressed ErbB-2 protein. However, contrary to this prediction, ErbB-2 homodimers that are driven either by a bivalent antibody (9) or by a point mutation (29) induce a mitogenic response that is weaker than that generated by ErbB-2-containing heterodimeric complexes. Another prediction made by the orphan receptor hypothesis is that the ErbB-2 ligand, if it exists, contains an EGF-like motif of six cysteine residues. However, it seems that no known EGF-like motif can directly bind to ErbB-2 with high affinity. For example, our most recent search for such an element in newly cloned EST databases identified one candidate, which we denoted NRG4 because the encoded protein exclusively binds ErbB-4 as its primary receptor (30). The EGF-like motif is found not only in ligand growth factors but also in cell adhesion proteins. For example, multiple copies of this domain are included in the extracellular matrix proteins laminin, tenascin, and thrombospondin, as well as in two *Drosophila* cell fate-determining proteins: Notch and Delta (reviewed in ref. 31). Our present results (Fig. 4), imply that all of the motifs included in proEGF, except the membrane proximal domain, belong to the second category of function. Indeed, modeling of the eight other motifs of proEGF, according to the published three-dimensional structure of EGF (32), indicated that domains 1–4 and 5–8 fall into distinct groups but that both groups significantly differ from the structures of EGF and NDF (M. Eisenstein, S.G., and Y.Y., unpublished results). Another important conclusion that emerged from the analysis of proEGF motifs is that the Gly-Xxx-Arg-Cys motif common to all ErbB ligands, but absent in nonligand motifs, may predict ErbB binding. Table 1 lists all of the currently known molecules that contain this motif, in the context of the EGF-like domain, along with their ErbB activating preference. Although it is clear that none binds to ErbB-2, it is also evident that signaling by all known ErbB ligands is enhanced by ErbB-2. This conclusion, along with the observation that certain anti-ErbB-2 antibodies can inhibit signaling by several NRGs and EGF-like ligands (Fig. 2), reinforces the possibility that ErbB-2 acts as a heterodimer partner rather than a direct receptor. Also supportive is the observation that each of the three other ErbB proteins serves as a direct receptor for more than one ligand (Table 1). It is therefore conceivable that, if ErbB-2 were able to bind a direct ligand, such a molecule would have been discovered, at least once.

Table 1. Receptor specificity of EGF-like ligands and neuregulins



All of the ErbB-stimulatory ligands are presented along with their ErbB preference. Interactions with the indicated ErbB homodimers (above diagonals) and the corresponding heterodimers with ErbB-2 (below diagonals) are indicated by using a color code: The most mitogenic interactions of each ligand are shown in black whereas white areas indicate absence of mitogenic signals. Note that ErbB-2 homodimers respond to no known ligand but that the mitogenic action of practically all growth factors can be augmented in the presence of ErbB-2. The data represent compliation of previous results obtained primarily with IL-3-dependent cells and the following ligands: NRG1s (9, 35), NRG2s (16), NRG3 (36), NRG4 (30), EGF (9, 22, 35, 37), transforming growth factor α (22, 37), epiregulin (15, 38), betacellulin (22, 39, 40), amphiregulin, and the viral ligands (17).

In the absence of an ErbB-2-specific ligand, it may not be practical to test the prediction that ErbB-2 acts solely as a receptor subunit. However, the presence of genes encoding EGF-like ligands in the genome of poxviruses provided us an attractive opportunity to test this possibility. Like ErbB-2-overexpressing human carcinomas, the skin lesions induced by poxviruses display epithelial hyperproliferation and a transformed phenotype (25). Because poxviruses underwent coevolution with their mammalian hosts and were selected for efficient induction of epithelial lesions, it is reasonable to assume that an ErbB-2 ligand, if it existed, would have conferred a significant selective advantage to poxviruses that encoded it. Therefore, the observation that none of the three known viral growth factors can directly interact with ErbB-2 (Fig. 5) implies that this receptor may not be able to accommodate a specific ligand. On the other hand, ErbB-2 seems to fulfil a similar role in viral infection to that played in human carcinomas; the observed specificity of SFGF and especially Myxoma virus growth factor to the most mitogenic heterodimer, namely the ErbB-2/ErbB-3 combination (Table 1), suggests that poxviruses, much like carcinogenic mechanisms, gained the ability to harness the signal amplification ability of ErbB-2.

Perhaps the best exemplification of the capacity of ErbB-2 to transactivate signaling initiated by ligands binding to other ErbBs is the ability to reconstitute an extremely strong mitogenic activity of ErbB-3, a receptor whose homodimers are inactive (9). Because ErbB-3 is expressed by many carcinomas at moderately high levels and ErbB-2 is ubiquitously expressed, the cooperation between the two receptors is thought to drive or maintain the transformed phenotype of epithelial tumor cells (33). Examination of the molecular mechanism underly-

ing ligand-induced formation of this heterodimer may provide an explanation to the role played by ErbB-2 (34): Apparently, ErbB-2 can bind at very low affinity ligands like NRG1, but only when they are presented to it by their primary receptors. This model predicts that ErbB ligands are endowed with two binding sites and that the lower affinity site preferentially recognizes the putative binding cleft of ErbB-2, which may be the target of class II mAbs (19).

In conclusion, ErbB-2 emerges as a master coordinator of a signaling network rather than as a receptor that mediates the action of one specific ligand. The relative topology of ErbB proteins, which are situated primarily on the basolateral face of epithelial cells, and their respective ligands, which are synthesized by the underlying stromal cells, implies that ErbB-2 can act as an amplifier of signaling by all of the stromal ligands listed in Table 1. Complete sequencing of the human genome and characterization of the remaining EGF motif-containing genes will ultimately answer the question whether this is the only function of ErbB-2 or whether a still-unknown ligand that binds to it with high affinity does exist.

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